This document contains several protocols for performing key steps in CoSMoS data analysis using imscroll. We thank Hailey Gahlon (University College, London) and Gengjing Zhao (MRC LMB, Cambridge) for putting together the initial version of these.

Note: The file ‘imscroll\_front\_panel.ppt’ shows images of the gui with the various buttons and menus numbered for reference in the instructions that follow. That file and several utility functions referenced in these instructions are in the …/imscroll/documentation directory of the Github repository. This file (‘CoSMos\_imscroll\_instructions.docx’) is also there.

Contents

[Setting up files and directories 1](#_Toc454744662)

[Setting up files that are necessary for using imscroll 2](#_Toc454744663)

[Starting imscroll 3](#_Toc454744664)

[Loading data 4](#_Toc454744665)

[Selecting and integrating AOIs 5](#_Toc454744666)

[Performing mappings 8](#_Toc454744667)

[Protocol for preparing a mapping file. 9](#_Toc454744668)

[Loading a mapping file. 14](#_Toc454744669)

[Performing a mapping 14](#_Toc454744670)

[Creating a driftlist 14](#_Toc454744671)

[Selecting control AOIs 17](#_Toc454744672)

# Setting up files and directories

To begin, directories should be established on the computer.

Matlab

avis

data

figfiles

mapping

figures

mfiles

images

driftlist

1. Go to c: drive and create a matlab folder
2. In the matlab folder create these corresponding folders: avis, data, figfiles, figures, mfiles and images
3. In the data folder create these two folders: driftlist and mapping
4. In the c:\matlab\figfiles folder insert the imscroll folder containing the Matlab scripts that is available online from the Gelles lab:

<https://github.com/gelles-brandeis/CoSMoS_Analysis>

5. Upload and save your TIFF data files in the images folder

6. Start Matlab and enter the following command to add your folder to the path used by Matlab when searching for files:

>>path(pathdef,genpath('c:\matlab\')) % This adds all the subfolders of the ‘matlab’ folder

% to the path searched by Matlab when attempting

% to locate a file or program that you call

>>cd c:\matlab

## Setting up files that are necessary for using imscroll ( it is necessary to do this only one time, specifically upon first installation of imscroll)

The imscroll program stores some intermediate parameters in \*.dat files that are contained in the

‘…\imscroll\gui\_files\’ directory created when imscroll was installed (step 4 above). The user must create initial copies of those \*.dat files by typing the command from within Matlab

>> rename\_ref\_to\_dat

The imscroll program will fetch and store files from locations specified through a user-created Matlab file, ‘filelocations.dat’ that contains a structure variable called ‘FileLocations’. The ‘filelocations.dat’ file is found in the ‘c:\matlab\figfiles\imscroll\gui\_files\’ directory (a subdirectory of the ‘imscroll’ folder that you should have already downloaded from github) . A ‘filelocaitons.ref’ Matlab file is already in that same ‘gui\_files’ directory to illustrate one example of the ‘FileLocations’ structure. If the ‘filelocations.dat file is not found upon startup, the program will not respond and the user will merely see error messages in response. Once created, the ‘filelocations.dat’ need only be accessed by the user to alter the locations where imscroll stores or fetches files.

As an example, to define and store the FileLocations structure the user types from within Matlab:

>>FileLocations.data =‘c:\matlab\data\’

>>FileLocations.avis =‘c:\matlab\avis\’

>>FileLocations.mapping = ‘c:\matlab\data\mapping\’

>>FileLocations.gui\_files = ‘c:\matlab\figfiles\imscroll\gui\_files\’

% And then save the file:

>>save c:\matlab\figfiles\imscroll\gui\_files\filelocations.dat FileLocations

Having these user-defined directories saves the user from constantly navigating directories to fetch and store files. The files defined in FileLocations are used for the following purposes.

**data**: Directory to store results such as lists of selected spot coordinates, integrated fluorescence traces, locations of detected spots and detected binding event intervals.

**avis**: Directory for avi files that may be created by the program. This feature is really obsolete in that we now use more flexible utility programs for this purpose.

**mapping**: Directory in which the program stores and retrieves files containing information for mapping AOI (areas of interest) coordinates.

**gui\_files**: Directory in which the program stores filelocations.dat, pre-set magnification values and transient lists of AOI coordinates.

## Starting imscroll

The program is called with a single argument ‘foldstruc’, but that argument is a structure that can specify multiple images files and one file with drift information. If your images are stored in stacked tiff files, then you for example supply the full path to the tiff files:

>>foldstruc.folder= 'c:\matlab\tiff\_files\B29p24a\_248\_fov1.tif '

>>foldstruc.folder2= 'c:\matlab\tiff\_files\B29p24a\_248\_fov2.tif '

>>foldstruc.folder3= 'c:\matlab\tiff\_files\B29p24a\_248\_fov3.tif '

>>foldstruc.folder4= 'c:\matlab\tiff\_files\B29p24a\_248\_fov4.tif '

>>foldstruc.folder5= 'c:\matlab\tiff\_files\B29p24a\_248\_fov5.tif '

Note that in the above definitions that the first member is NOT numbered (foldstruc.folder), but all files past the first member are numbered (foldstruc.folder2, etc. and the program allows up to 5 input tiff files).

If you are instead using GLIMSE image files (uncompressed binary files output by the LabView acquisition program used in our lab) you specify only the directory containing the GLIMSE image file. For example,

>>foldstruc.gfolder= 'P:\image\_data\August\_10\_2015\b31p46b\_074\'

>>foldstruc.gfolder2= 'P:\image\_data\August\_10\_2015\b31p46c\_075\'

>>foldstruc.gfolder3= 'P:\image\_data\August\_10\_2015\b31p47a\_080\'

>>foldstruc.gfolder4= 'P:\image\_data\August\_10\_2015\b31p47b\_081\'

>>foldstruc.gfolder5= 'P:\image\_data\August\_10\_2015\b31p47b\_086\'

Again, note in the above definitions that the first member is NOT numbered (foldstruc.gfolder), but all files past the first member are numbered (foldstruc.gfolder2, etc. and again the program allows you to specify up to 5 GLIMPSE files). Once started, the imscroll program will allow you to switch between any of the files you have specified using the foldstruc structure.

If you have information specifying the stage drift, that may be input as well using

>>foldstruc.DriftList=driftlist;

The format of the driftlist variable specified above is:

[ (image frame number) (Δx increment for that frame) (Δy increment) (time)]

so the first few lines of the driftlist matrix might look like:

>>driftlist(1:5,:)

= 1.0000 0 0 7.8184E7

2.0000 -0.0056 0.0247 7.8185E7

3.0000 0.0112 -0.0279 7.8186E7

4.0000 -0.0219 0.0125 7.8187E7

5.0000 0.0833 0.0007 7.8188E7

The ‘time’ column need not be included (it is used in other programs), and in the above example it is written in milliseconds as specified by the clock accessed within LabView.

The program is then started by typing

>>imscroll(foldstruc)

# Loading data

Tiff data files can be loaded by typing commands in Matlab.

1. Start Matlab and enter the following commands:

>>path(pathdef,genpath('c:\matlab\'))

>>cd c:\matlab

>>[fn fp]=uigetfile

% A dialog box opens through which you should navigate to and select the tiff data file you saved in the ‘images’ folder (note: in the dialog box that opens you might need to select ‘all files’ from the dropdown menu of the ‘files of type’ box if nothing is appearing)

>>foldstruc.folder=[fp fn]

>>imscroll(foldstruc)

% A Matlab GUI opens with your data

1. If you want to load more than one file, type the commands:

% Get the second file and place it in folder2

>>[fn fp]=uigetfile

>>foldstruc.folder2=[fp fn]

% Get the third file and place it in folder

>>[fn fp]=uigetfile

>>foldstruc.folder3=[fp fn]

You may select up to 5 total image sequence files

for simultaneous access from within the imscroll program.

# Selecting and integrating AOIs

Areas of interest (AOIs) can be selected in the imscroll GUI.

1. The contrast/brightness of the display starts up in an automatic mode that scales the display according to the minimum and maximum intensity contained in each image. It is frequently more usefule to set this maunually. To switch to manual scaling: In the imscroll GUI click on Autoscale (#31) to go to Manual Scale at which time two slider controls appear that enable the user to set the minimum (black) and maximum (white) display intensity. The maximum of both sliders is initially set to 1000, but this often needs to be changed. To alter the maximum of the slider place the cursor in the text region #29 and type the maximum intensity for the slider control (it must be greater than the current value of the slider or the slider control will disappear and an error will appear in the command window). A maximum of value of 2000 is a common setting.
2. When viewing tiff files, in the dropdown menu #41 select ‘Folder’ and use the plus and minus signs above it to select the file that want to look at (1 corresponds to the file contained in foldstruc.folder, 2 to that in foldstruc.folder2, etc)
3. Use the scroll bar (#2) to move through the frames
4. You may switch between full view of the sample or to a magnified image by clicking button #32. When operating in ‘magnified’ mode you may select from numerous magnified regions from the dropdown menu #33. The up ‘^’ and down ‘v’ arrows adjacent to #33 also navigate between the choices. All the magnified regions are user-setable and will be remembered when the program is exited and later restarted. To select a sub-region for magnification first click on button #35, then follow that with two clicks (left mouse button) within the image #1 at locations defining the two diagonal corners of the desired region. Click Full Screen/Magnified on button #32 to zoom in and out of the field of view. You may also manually define a magnified region using the text region #34.
5. Averaging of frames is done by editing text #8 by either direct typing or using buttons #9 and #10.
6. Jumps between displayed frames may be performed using buttons #4 and #5 with the direction (increment or decrement) set by the toggle button #3. The jump by button #6 is user-setable by the follwing steps (example for setting button #6 to a jump of 3 frames): Edit region #8 to read ‘3’, select ‘Set Variable Jump’ from the dropdown menu (#55) , then click on the GoButton #54.
7. To select a new collection of individual AOIs, press #23 and repeatedly left click within the image region #1 at the desired locations to mark new AOIs (any AOIs existing previously will vanish). When done , right click.
8. Individual AOIs can be removed with Remo (#44) or added with Add AOIs (#45). To start over with a new set of AOIs use button #23 (see 7 above).
9. To select a set of AOIs automatically, use Auto Spot Picking (#13-#17). The ‘Noise diam’ (#17) is usually set to 1 pixel and we find that setting ‘Spot diam’ (#18) to 5 pixels works well for our images. Vary ‘Spot Brightness’ #15 to best select the fluorescent spots in the image. A ‘Spot Brightness’ setting too low will detect spurious spots arising from noise while a setting too high will fail to detect legitimate fluorescent spots that appear in the image. Only spots within the region appearing within the image window #1 will be selected. Spots outside the region visible (due to viewing the image under magnification) will not be subjected to spot selection.
10. Change the pixel size for AOI area selection using #26 as needed
11. To see AOI numbering write the AOI number in region #46 or change the number in the #46 region using the +/- increment/decrement buttons adjacent to region #46.
12. To move a single AOI click Move1 button (#49) (followed by two clicks within the image #1: first click to select the AOI to move, second click at the new location of that AOI. To move all the AOIs click button (#47) and use the buttons that appear to translate all AOIs in both x and y directions. The number of pixels moved for each button press is the number appearing in text region #46 (which is user settable)
13. Select Save AOI Information (#55) to save the positions of the AOIs, which will be saved as a default.dat file in the c:\matlab\data\ folder. That file should be renamed by the user for later reference (it will otherwise be overwritten). The file contains a matrix variable ‘aoiinfo2’ that lists the AOI data as follows:

1 2 3 4 5 6

[ Frame FrameAve x-coord y-coord AOI size AOI#]

The file containing the aoiinfo2 variable may be loaded into the command window for later use,

and may also be loaded back into the imscroll program, as outlined below.

1. To import an AOI list (containing the aoiinfo2 matrix) into the imscroll program paste the filename containing the AOI list (i.e. containing the aoiinfo2 matrix) into text region #53. Next, select ‘Load Fitdata:marking spots’ from the dropdown menu (#55) and click the GoButton #54. Later on (below) we describe how to integrate these AOI regions or fit the spots therein with a Gaussian function. When that is done a file is created that contains an ‘aoifits’ structure variable. If you just want to load AOI coordinates from a file containing such an ‘aoifits’ structure you place the name of that file into the text region #24, select ‘Load AOIs’ from the dropdown menu (#55) followed by a click on the GoButton #54.
2. AOIs may be centered on the fluorescent spots contained therein using a Gaussian fit. First, adjust the AOI size using text region #26 so the spots are fully contained within the AOI. Also, note the current frame number from text region #7 and write that number in the text region #25. To then center AOIs, select ‘Gauss2d+Int’ from the dropdown menu (#19) and press the ‘Fit AOIs’ button (#22). Another gui screen opens up, and that new gui may be immediately closed (a ‘c:\matlab\data\default.dat’ file has now been automatically written containing the Gaussian fit data). Then, select ‘Centering’ from the dropdown menu (#55) and click on GoButton (#54). The program uses the Gaussian fit data from the new ‘c:\matlab\data\default.dat’ written file to center the AOIs and you may notice a slight shift in AOI positions as a result of this centering.

16. (integrated fluorescence) vs (frame number) traces may be created and stored by integrating the fluorescence intensity contained within each AOI over a user-specified frame range of the image sequence. To integrate the AOIs, specify the frames that you want to process in the text region (#25), e.g. [1:500] for frames 1 to 500 (including the [ ] brackets is required) and then select ‘Int. linear interp’ from the dropdown menu (#19). Select Fixed AOI (#18) in the absence of a driftlist and Moving AOI (#18) when you want to apply a driftlist (see section E: Creating a driftlist). Click Fit AOIs button (#22). The progress of the integration may be monitored in the command window

1. Once the integration is complete, another gui window will open (Plotargs). After integration a c:\matlab\data\default.dat’ file is saved automatically and that file contains all the integration data in a structure named ‘**aoifits**’. . To retain the integrated data for later reference and use, the user should rename the ‘default.dat’ file (the file will otherwise be overwritten when the program again stores information). The user may view the integrated AOI traces from within the newly opened Plotargs GUI, To view the integrated traces select ‘int. aoi’ from the dropdown menu #39, press the button #40 to toggle on, and then move up and down through the AOI trace number specified by the text region #41 using the +/- increment/decrement buttons. The AOI number corresponds to that in the imscroll GUI.
2. There are two plot regions (middle and bottom) for comparing (integrated intensity) vs (frame number) traces. Data from other integrations may be loaded into the gui and displayed. That data should be contained in files that reside in the c:\matlab\data\ directory. Write the filename containing **aoifits** data into text region #12 and click button #11 for display on the middle axis. Likewise, write the filename containing **aoifits** data into text region #18 and click button #17 for display on the bottom axis.
3. Images of the AOI regions corresponding to different frames along the trace may be viewed in the top window of the gui. The user must specify the files from which the images will be retrieved. If using stacked tiff files, set the dropdown menu #8 to ‘Tiff Folder’. Click the load Image File 1 button (#9) (opens a dialog box) and navigate to the tiff file containing the images that correspond to the AOIs and traces displayed in the middle plot window. Likewise, click the load Image File 2 button (#10) and navigate to the tiff file containing the images that correspond to the AOIs and traces displayed in the bottom plot window. To view gallery images, select the Click button(#23) and left click on a point along the int. aoi trace in the bottom plot to view gallery images of the spots that correspond to that part of the trace. The position of the vertical line defines the image at the center of the set of spot images. The images appear as multiple rows of dual images (default is three rows of 13 images each). In the dual image rows, the top image corresponds to the AOI and file displayed in the middle plot window, while the bottom image corresponds to the AOI and file displayed in the bottom plot window. If using a calculated driftlist to correct for stage drift (#18 dropdown menu in the imscroll gui must then be set to ‘Moving AOI’) the gallery images will use that correction and present images for the AOIs that have been translated according to the driftlist. When viewing gallery images, the variable jump button #6 in the imscroll gui should be set to 1 frame (otherwise, the successive gallery images displayed will progress by more than 1 frame).
4. The text region #5 contains parameters that determine how the gallery images are presented. The values in text region #5 correspond to [(frame begin) (frame end) (#images displayed horizontally) (# of image rows displayed vertically)]. The (frame begin) and (frame end) entries show the frame range following a user click on a trace, and they may also be manually set and then displayed by clicking on the #3 ‘Display AOIs’ button. The (#images displayed horizontally) and (# of image rows displayed vertically) entries may be user set to alter the arrangement and number of the gallery images. The size of each gallery image is set by the text region #26 in the imscroll gui. For example, if that text region contains the number 5 each gallery image will display 2\*5+1 on a side, or 11 x 11 pixels.
5. The size of each gallery image is set by the text region #26 in the imscroll gui. For example, if that text region contains the number 5 each gallery image will display 2\*5+1 on a side, or 11 x 11 pixels.
6. The display window #51 may be used to view a magnifiable region around one AOI from the bottom trace (#47 set to 2) or the middle trace (#47 set to 1). Controls #45 allow increment/decrement of the displayed frame, and #49 controls the magnification scale.
7. The contrast/brightness of image region #56 and #51 is set by the sliders #52 (minimum intensity) and #54 (maximum intensity). To use the sliders for this purpose the user must first click the radio button ‘Display Scales’ #57. The maximum value for the sliders is set by the user typing in text region #53.

# Performing mappings

Mapping is performed in order to correlate spot locations recorded in separate channels (e.g. images recording different color fluorescence emissions). The method and descriptive protocol for mapping is described in Methods. 2015 Sep 15;86:27-36 (doi: 10.1016/j.ymeth.2015.05.026). It is recommended that a user read that description before going forward here.

The goal in forming a map is to assemble a list of N paired locations in the two channels (), where the pairs correspond to the same sample location in the two images. The spot pairs are selected many at a time, and the final map usually contains a list of a few hundred paired calibration AOIs. Once the list of calibration spots are recorded the user can map between sites in channel 1 and the corresponding site in channel 2 (e.g. use the sites of DNA designated by fluorescent spots in channel 1 to monitor landings of a protein labelled by a dye that emits at a different color).

## Protocol for preparing a mapping file.

It is recommended that users consult the article in Methods 86:27 (2015) for a description and use of mapping.

To map a list of AOIs (areas of interest) from Field 1 to Field 2 (e.g. two images of the same sample region--perhaps with a dual image setup as in Biophys J 91:1023 (2006) - - recorded with different color excitation, or two images of the same sample region before and after a lane flush that results in a slight displacement) we require use of a calibration mapping file. We typically construct this mapping file by preparing a slide sample in which we have surface-anchored oligonucleotides that are each labeled with multiple dyes of different color. We will build up a mapping file by

picking co-localized spot pairs in Field1 and Field2 (e.g. images recorded for different color dyes) in several sample regions and repetitively adding them to a growing list of paired spots for a progressively better mapping file.

Initially, we have no mapping file and we need to just translate and align the spots from one small region of the field to the other by using the ‘move’ feature in imscroll (Button #47 in imscroll gui). Alternatively, the user could use an old mapping file at the outset, or just build a crude map by manually choosing a few individual spots in a FOV using the mapping sub-gui (opened by clicking button #27 in imscroll gui).

**I. First round of defining spot pairs to the calibration mapping list.**

**-Auto pick spots in the Field1 (e.g. red field)** % Set the threshold high enough to

% avoid picking spurious spots due

% to noise. The fluorescent spot

% AOIs (areas of interest) are then

% marked by a box surrounding

% each spot

🡪Use controls #13-18 in the ‘Auto Spot Picking’ panel, as described previously

**-remove inappropriate AOIs** % Remove overlapping spots or obvious dirt

🡪 Set dropdown menu #20 to ‘Remove Close AOIs’, set the text region that then appears to e.g. 6 (pixels) and click button # 20. You may follow this by a quick visual examination of the remaining spots during which you remove instances of two spots erroneously contained within a single AOI box and obvious instances AOIs containing just surface dirt.

**-gaussian refine positions (centering spots)**

**🡪**Use the spot centering operation described under ‘Selecting and Integrating AOIs’ above.

**-move spots to Field2** % use manual translation, crude mapping,

🡪This first mapping of Field1 to Field2 usually occurs using data from your most recent map. When no approximate map exists the user usually starts by picking spots within a small region of Field1 (no larger than 10-15 microns in diameter) and manually translating them into Field2 using the ‘Move’ feature (button #47, see above in ‘Selecting and Integrating AOIs’) to achieve the best overlap between the Field1 AOIs and the Field2 spots. The resulting map may be progressively expanded into larger areas for each round of picking additional spots.

**-remove inappropriate AOIs** % empty AOIs, neighbor AOIs that are too

% close, dirt etc

🡪Some of the AOIs from Field1 will be empty when moved to Field2. To remove those empty AOIs set the dropdown menu #20 to ‘Remove MT AOIs’, set the text region that then appears to maybe 2 or 3 pixels (maximum distance between AOI center and any spot) and then click button #21. Additional AOIs from Field1 that do contain Field2 spots but are nonetheless too close to other Field2 neighbors may be removed manually (button #44, see above).

**-Gaussian refine (centering spots within AOIs)**

**🡪**Center the fluorescent spots in their AOI boxes. The procedure is already described above.

**-Define Field 2** % popup menu in mapping panel

🡪The AOIs remaining in Field2 are now centered on Field2 fluorescent spots, they have corresponding partner spots in Field1 and do not have any encroaching neighbors in either Field2 or Field1. We therefore designate them as the first AOIs in our list of calibration AOIs. To do this set dropdown menu #20 to ‘Define Field2’ and click on button # 21. We next have to move back to Field1 and associate these Field2 AOIs with their corresponding partners to these Field2 AOIS.

**-move spots back to Field 1** % use manual translation, crude mapping etc

🡪We move the Field2 AOIs back to Field1 where they all should contain Field1 fluorescent spots. This movement or mapping is performed again using the crude existing map from a prior calibration or using the ‘Move’ button #47.

**-Gaussian refine (centering spots within AOIs)**

**🡪** Center the fluorescent spots in their AOI boxes. The procedure is already described above.

**-Define Field1** % popup menu in mapping panel

🡪 Set the dropdown menu #20 to ‘Define Field1’ and click on button #21. This designates each of these locations in Field1 as the matching pair to the Field2 locations recorded in the **Define Field2** step above.

**-Make Map** % popup menu in mapping panel. This creates a

% fitparms.dat mapping file in the

% mapping directory and updates the mapping

% parameters in imscroll

🡪 We now have a paired set of calibration mapping spots in both Field1 and Field2. A map may be saved and simultaneously loaded into imscroll for ongoing use and refinement. To do this set the dropdown menu #20 to ‘Make Map’ and click on button #21.

The user will now see three figures appear. Figure 23 plots the (x y) location of all the AOIs in your map. Figures 22 and 23 are created to help identify mapping calibration AOIs that have been erroneously chosen (mis-pairing spots in Field1 and Field2).

The mapping list consists of paired Field1/Field2 sites. To create Figures 22 and 23 the (x y) coordinates of each Field1 AOI is mapped into Field2 using the remaining pairs of the mapping list. Those Field2 mapped coordinates are then compared to the Gaussian-fit (x y) coordinates of the actual paired Field2 fluorescent spot. The difference between the mapped and Gaussian fit coordinates appears in Figure 22 (for x) and Figure 23 (for y). That difference should be dominated by the localization accuracy of the Gaussian fits (typically a few tenths of a pixel). When enough correctly paired Field1/Field2 spots are in the list, any incorrectly paired spots will show up as outliers in these plots. Typically, we then edit the mapping list by removing any AOIs appearing in Figure 22 or Figure 23 with differences larger than e.g. 0.4 pixels.

**-Edit the Mapping points: remove AOIs that that have been erroneously picked**.

🡪Remove AOIs with outliers in the x coordinate: Set the dropdown menu #20 to ‘Remove X2 AOI’ and click the button #21. A crosshair will appear in Figure 22 and the user may continue to left-click on outlier points to remove the corresponding AOI from the mapping calibration list. When finished, user should right-click to end that editing mode.

Remove AOIs with outliers in the y coordinate: Set the dropdown menu #20 to ‘Remove Y2 AOI’ and click the button #21. Repeat the editing process Figure 23.

A list of the remaining paired spots is in the mapping file that has been automatically stored as ‘c:\matlab\data\mapping\fitparms.dat’ (i.e. the fitparms.dat file has been stored within the mapping directory specified in the c:\matlab\figfiles\imscroll\gui\_files\filelocations.dat file ( see the description in the ‘imscroll\_setup\_and\_startup.doc’ file). Additional pairs may be now added to this mapping list (the fitparms.dat file will be overwritten), so for safety it is recommended that the fitparms.dat file be renamed now and after each round of adding points to the list.

The protocol for adding more pairs to the mapping list is very similar to the above. It differs when the user choses to ‘add to Field1’ in place of the ‘define Field1’ step above.

**II. Adding additional spot pairs to the calibration list**

**-change frame within imscroll and view a new field-of-view with different spots**

**-auto pick spots in Field1**

**-remove inappropriate AOIs**

**-Gaussian refine (centering spots within the AOIs)**

**-map spots to Field 2** % operations under **I** above have made a crude map

% which we now use to map our spots

🡪Be certain that the ProxMap button #12 is toggled to the ‘on’ position (see section on ‘Performing Mappings’). Set dropdown menu #55 to ‘Map AOIs (out: x2y2)’ and click on button #54.

**-remove inappropriate AOIs**

**-Gaussian refine (centering spots within the AOIs)**

**-Add to Field 2**

🡪The AOIs remaining in Field2 are now centered on Field2 fluorescent spots, they have corresponding partner spots in Field1 and do not have any encroaching neighbors in either Field2 or Field1. In this step we add these new AOIs in Field2 to the list of existing Field2 AOIs. To do this set dropdown menu #20 to ‘Add to Field2’ and click on button #21.

The image region #1 now shows *all* the AOIs in Field2 (the new ones just added plus those earlier placed into the list), but going forward we need to briefly work only with the new AOIs that were just added. The next step is needed in order to restrict upcoming operations to just the new Field2 AOIs.

**-Restore preAddition AOIs** % popup menu in mapping panel. This restores the

% set of AOIs to those just before

% the‘Add to Field 1’ command immediately above

🡪 Set dropdown menu #20 to ‘Restore preAddition’ and click on button #21. All the prior Field2 AOIs will disappear from the image in #1, leaving only the new set of AOIs that were just added to the list.

**-map spots to Field1**

🡪Be certain that the ProxMap button #12 is toggled to the ‘on’ position (see section on ‘Performing Mappings’). Set dropdown menu #55 to ‘inv map AOIs (out: x1y1)’ and click on button #54.

**-Gaussian refine (centering spots within the AOIs)**

**-Add to Field 1** % popup menu in mapping panel. Adds these

%Field 1 spots to those prior Field 1 spots,

% increasing the number of AOIs in Field 2.

% All of the Field 2 AOIs are now displayed.

% The number of Field 2 AOIs now matches the

% number of Field 1 AOIs

🡪 this set dropdown menu #20 to ‘Add to Field1’ and click on button #21.

**-Make Map** % popup menu mapping file. This overwrites the

% fitparms.dat mapping file in the

% mapping directory and updates the mapping

% parameters in imscroll

🡪As above, set the dropdown menu #20 to ‘Make Map’ and click on button #21.

**-Edit the Mapping points: remove AOIs that that have been erroneously picked**.

-**repeat the steps starting at II until an adequate mapping file is made (usually containing several hundred spot pairs).**

**🡪**Again, it is recommended that the user rename the ‘c:\matlab\data\mapping\fitparms.dat’ file after each round of adding mapping pairs to insure that the list remains intact.

For loading previously-created mapping files into the imscroll gui see ‘Loading a mapping file’ below.

## Loading a mapping file.

Maps are loaded in imscroll by choosing dropdown menu #55 to be ‘Load Fitparms’, placing the name of the mapping file into the text region # 53 (that mapping file must be in the mapping directory designated by your filelocations.dat file, as described above) and then clicking on the GoButton #54. **Note**: User should then click on the ‘ProxMap’ button #12, which toggles it into the on position, ensuring that proximity mapping is being used—see description of proximity mapping in Methods. 2015 Sep 15;86:27-36).

## Performing a mapping

Performing a mapping of AOI sites that have been selected (and a map has been loaded):

To map from Field1 to Field2: Set the dropdown menu #55 to ‘Map AOIs (out: x2y2)’ and click the GoButton # 54. To map from Field2 to Field1: Set the dropdown menu #55 to ‘inv map AOIs (out: x1y1)’ and click the GoButton # 54. (**Note:** when the ProxMap botton is properly toggled to the on position, the user should note several seconds of elapsed time between clicking the GoButton #54 and the completion of the mapping operation)

# Creating a driftlist

Experiments can last for up to an hour or more, and over that time interval the microscope stage is subject to thermal and mechanical drift. The imscroll program can correct for that drift by displacing the AOI (x y) centers throughout an image sequence. This insures that spot integrations or Gaussian fits are always performed on spots that are centered within the AOIs. The driftlist used by the program is an Nx4 matrix (N=# of image frames in the sequence) of the form:

[(frame #) Δx Δy (time in millisec) ]

where e.g. for frame 37 the Δx and Δy list the small stage drift that occurred between frames 36 and 37.

Once this driftlist exists it may be included in the input structure of imscroll (see imscroll\_setup\_and\_startup.doc) using:

>>foldstruc.DriftList = driftlist;

Once imscroll has been started the user needs to set dropdown menu #18 to ‘Moving AOI’, and any AOIs will be displaced whenever the user alters the image frame being viewed. The locations for AOIs integrated, Gaussian fit or viewed as gallery images within the plotargs gui (see above in **Selecting and integrating AOIs**) will also be subject to the same drift correction.

To create a driftlist the user will first Gaussian fit (tracking the (x y) center location) one or more fluorescent spots that are present (when combined) for the entire image sequence. Fluorescent beads provide good fluorescent spots in that there is no blinking involved. In that instance a user can best Gaussian fit that bead throughout the image sequence by clicking on the ‘track AOIs’ radio button next to the #18 popup menu. As the bead spot is Gaussian fit throughout some frame range, the (x y) center result of one frame is then used as the starting guess for fitting the next frame. This may not be used for single dye spots that blink. In that instance it often requires that multiple fixed-position AOIs be used (offset from one another) to encompass a spot for fitting throughout an image sequence (the spot drifts out of a single fixed-position AOI box).

In the following protocol it is assumed that the user has already used imscroll to Gaussian fit some reference spots throughout the necessary frame range, saving those results in a file that contains the ‘aoifits’ structure created by the program. Note that the aoifits structure has a member (aoifits.data) containing all the fit parameters (amplitude, (x y) centers, offset) for all the AOIs in a single matrix. The first few steps in the protocol rearranges that two dimensional aoifits.data matrix into a three dimensional matrix, where now the third dimension runs over individual AOIs.

Also, the user must supply a ‘vid’ variable in which the ‘vid.ttb’ member is the time base for an image file. That is, length(vid.ttb)=(number of frames in the image file). For the ‘construct\_driftlist\_time\_v1( )’ routine the time base is that for the image file used for Gaussian-fitting the reference spots. For the ‘driftlist\_time\_interp( )’ routine the time base is that for the image file for which the driftlist is produced. Those two image files will usually, but not always be the same. For example, the Gaussian fit spots might track dye-labeled DNA with images recorded only once per minute while the driftlist is prepared for a file recorded continuously at 1 frame/s in between those images of the dye-labeled DNA. The ‘driftlist\_time\_interp( )’ program can then create that finer grid driftlist by interpolating between the sparse drift information from the dye-labeled DNA tracking.

The steps for a driftlist include (a) picking AOIs that persist over a stack (b) doing a Gaussian fit and (c) making a cell array for the Gaussian spots. Usually approximately five or fewer spots are used for drift correction.

**Instructions for constructing driftlist**

In this example we have already Gaussian fit three spots through part of an image sequence. The stage drift is then tracked through the changing (x y) coordinates of the three spots. In this instance no single spot survived through the entire experiment due to photobleaching. The (x y) track of each spot is therefore used only for a subset of the frames in the image sequence (frames 1 to 47 for spot 1, 1 to 300 for spot 2 and 250 to 500 for spot 3), and the tracks are pieced together by the programs to obtain the drift throughout the frame range of interest (frames 1 to 500). Note also that here the drift is corrected only for the first 500 frames of an image sequence of total length 7595 frames.

load c:\matlab\data\exampleaoifits.dat –mat % Load the ‘aoifits’ structure stored

% by imscroll for Gaussian-tracked

% spots

dat=draw\_aoifits\_aois\_v1(aoifits,'y'); % Split the aoifits.data matrix into

% individual matrices (now one matrix for

% each AOI rather than all AOIs in the

% single aoifits.data matrix

help construct\_driftlist\_time\_v1 % First function used for making driftlist

% This will display use instructions for the

% function

xy\_cell{1}.dat=dat(:,:,1); % Make the cell arrays for the Gaussian spots

xy\_cell{2}.dat=dat(:,:,2); % used in making the driftlist

xy\_cell{3}.dat=dat(:,:,3); % Data for the xy locations of the spots in each frame

xy\_cell{1}.range=[1 500]; % Total Range of tracking the spots. That is, the

% initial aoifits structure (and the dat matrix)

% contains data only for the first 500 frames of the

% image sequence

xy\_cell{2}.range=[1 500];

xy\_cell{3}.range=[1 500];

xy\_cell{1}.userange=[1 47]; % Range that the data that is used (rest of the data is

xy\_cell{2}.userange=[1 300]; % ignored) for each of the 3 spots

xy\_cell{3}.userange=[250 500];

[fn fp]=uigetfile % Get the glimpse sequence file name and path

eval(['load ' [fp fn] ' -mat']) % (file in which we tracked the spots). Here we are just

% retrieving the file containing the vid structure with the

% vid.ttb member time base. For a stacked tiff file the user

% must also define a time base called vid.ttb for use in the

% ‘construct\_driftlist\_time\_v1’ and ‘driftlist\_time\_interp’

% functions

SequenceLength=7595; % Total # of frames in the glimpse file. That is, we drift

% correct only the first 500 frames of a 7595 frame length

% file

CorrectionRange=[1 500]; % Frame range over which we correct the drift

% Next, run function that constructs a cumulative drift vs (time) plot

drifts\_time=construct\_driftlist\_time\_v1(xy\_cell,vid,CorrectionRange,SequenceLength,[4 4],[2 11 2 11]);

% In the above function call the last two parameters ( ..[4 4], [2 11 2 11])

% deal with smoothing the noise in the driftlist track. See the function

% header for a more details. For a driftlist without any smoothing the last

% argument may be set to [2 3 2 3].

% Run function that constructs drift vs frame number for our file (the

% vid.ttb time base here need not match the vid.ttb in the step preceding,

% but it must span a subset of the time in that above step).

drifts=driftlist\_time\_interp(drifts\_time.cumdriftlist,vid);

foldstruc.DriftList=drifts.diffdriftlist; % Assign driftlist to proper member

% member of foldstruc structure

driftlist=drifts.diffdriftlist; % Also assign driftlist just for saving

% Save all the stuff used to make the driftlist

save c:\matlab\data\driftlistparameters.dat xy\_cell SequenceLength CorrectionRange vid

% Save the driftlist in easy-to-remember file. Next time you need this driftlist you need

% only load the example\_driftlist.dat file and then set foldstruc.Driftlist=driftlist

save c:\matlab\data\driftlists\example\_driftlist.dat driftlist

# Selecting control AOIs

Experiments usually record binding events (e.g. appearance of dye-labelled proteins) at sites of substrates (e.g. dye-labelled DNA, RNA or proteins immobilized on a slide surface). Events on control AOIs are used to distinguish between specific and nonspecific events. For this purpose we select AOIs that intentionally do NOT contain substrate molecules (e.g. dye-labeled DNA). Any landings that occur at such sites are then attributable to background nonspecific events. The statistics of bindings at specific sites necessarily includes a nonspecific background contribution, and those specific binding statistics must be corrected using the data measured at the control AOIs.

To select contol AOIs we set up a close-packed grid of AOIs, then eliminate any such AOI that is close to a fluorescent substrate molecule. The operation of picking the nonspecific AOIs is thus conducted using an image that contains the fluorescent spots of the substrate molecules that will be used as specific target sites in the experiment.

1. Set the threshold for spot brightness #15 at a level sufficient to detect the fluorescent spots arising from the substrate molecules.

2. Use the text region #26 to set the AOI size to e.g. 10 pixels.

3 Click the ‘AOI grid’ button (#37) and a crosshair will then appear in the image #1. Draw a boundary for the region that will contain the contol AOIs, typically the same region that contains the fluorescent substrate molecules. See Figure 9 in Methods 86:27 (2015).

4 Change the frame by clicking button #5 to display the resulting grid of AOIs throughout the defined region. The size of the AOIs is set by the pixel size previously written into the text

region #26.

5 We now want to remove all the AOIs that are close to any fluorescent substrate site. Select ‘Remove Spot AOIs’ #20 and set the text region that appears to read e.g. 7 pixels. Click on button #21 to then remove all AOIs within 7 pixels of a detectable spot. Additional spots may be remove manually using button #44 (usually not necessary).

The AOIs that remain do not contain any fluorescent substrate molecule and may be used as your set of control AOIs. The settings for pixel size and spot proximity may be modified to alter the number of AOIs in this set.

6. To save this list of control AOIs, set the popup menu #55 to ‘Save AOI Information’ click the button #54. Rember to rename the resulting ‘default.dat file (containing an aoiinfo2 matrix listing of the control AOI coordinates) for permenant storage.